American Physiological Society | Journal of Applied Physiology

PUBLISHED ARTICLE
ARCHIVES
SUBSCRIPTIONS
SUBMISSIONS
CONTACT US

J Appl Physiol (1985). 2018 Nov 1; 125(5): 1475–1481.
Published online 2018 Aug 16.
doi: 10.1152/japplphysiol.00625.2018: 10.1152/japplphysiol.00625.2018

PMCID: PMC6295482
PMID: 30113272

Measurement of nitrate and nitrite in biopsy-sized muscle samples using HPLC

Ashley D. Troutman,[1,2] Edgar J. Gallardo,[1] Mary Beth Brown,[2] and Andrew R. Coggan[1,3]

[1]Department of Kinesiology, Indiana University-Purdue University Indianapolis, Indianapolis, Indiana
[2]Department of Physical Therapy, Indiana University-Purdue University Indianapolis, Indianapolis, Indiana
[3]Department of Cellular and Integrative Physiology, Indiana-University Purdue University Indianapolis, Indianapolis, Indiana

Corresponding author.
Address for reprint requests and other correspondence: A. R. Coggan, Dept. of Kinesiology, Indiana Univ.-Purdue Univ. Indianapolis, IF 101C, 250 University Blvd., Indianapolis, IN 46202 (e-mail: acoggan@iupui.edu).

Received 2018 Jul 10; Revised 2018 Aug 2; Accepted 2018 Aug 15.

Abstract

Studies of rats have indicated that skeletal muscle plays a central role in whole-body nitrate (NO_3^-)/nitrite (NO_2^-)/nitric oxide (NO) metabolism. Extending these results to humans, however, is challenging due to the small size of needle biopsy samples. We therefore developed a method to precisely and accurately quantify NO_3^- and NO_2^- in biopsy-sized muscle samples. NO_3^- and NO_2^- were extracted from rat soleus samples using methanol combined with mechanical homogenization + ultrasound, bead beating, pulverization at liquid N_2 temperature or pulverization + 0.5% Triton X-100. After centrifugation to remove proteins, NO_3^- and NO_2^- were measured using HPLC. Mechanical homogenization + ultrasound resulted in the lowest NO_3^- content (62 ± 20 pmol/mg), with high variability [coefficient of variation (CV) >50%] across samples from the same muscle. The NO_2^-/NO_3^- ratio (0.019 ± 0.006) was also elevated, suggestive of NO_3^- reduction during tissue processing. Bead beating or pulverization yielded lower NO_2^- and slightly higher NO_3^- levels, but reproducibility was still poor. Pulverization + 0.5% Triton X-100 provided the highest NO_3^- content (124 ± 12 pmol/mg) and lowest NO_2^-/NO_3^- ratio (0.008 ± 0.001), with the least variability between duplicate samples (CV ~15%). These values are consistent with literature data from larger rat muscle samples analyzed using chemiluminescence. Samples were stable for at least 5 wk at −80°C, provided residual xanthine oxidoreductase activity was blocked using 0.1 mmol/l oxypurinol. We have developed a method capable of measuring NO_3^- and NO_2^- in <1 mg of muscle. This method should prove highly useful in investigating the role of skeletal muscle in NO_3^-/NO_2^-/NO metabolism in human health and disease.

NEW & NOTEWORTHY Measurement of nitrate and especially nitrite in small, i.e., biopsy-sized, muscle

samples is analytically challenging. We have developed a precise, accurate, and convenient method for doing so using an affordable commercial HPLC system.

Keywords: high performance liquid chromatography, human muscle, nitrate, nitric oxide, nitrite

INTRODUCTION

Skeletal muscle has recently emerged as a central player in whole-body nitric oxide (NO) metabolism. Specifically, studies of rodents by Piknova et al. (7, 22, 23) have demonstrated that, due in part to its mass, muscle represents the largest reservoir of nitrate (NO_3^-) in the body. This NO_3^- can potentially be reduced intramuscularly to form nitrite (NO_2^-) and then NO via mammalian NO_3^- reductases [e.g., xanthine oxidoreductase (XOR)], thereby possibly contributing to, e.g., exercise hyperemia (22). Alternatively, NO_3^- can be exported from muscle via the circulation, thus possibly supporting NO formation in other tissues. In turn, muscle seems to depend upon both endogenous and exogenous sources to maintain its stores of NO_3^- and NO_2^-. In particular, NO synthase 1-deficient mice exhibit a dramatic reduction in muscle, but not liver, NO_3^- concentration (23), demonstrating the importance of oxidation of locally produced NO in maintaining this pool. On the other hand, feeding rats a low NO_3^-/NO_2^- diet for 7 days also results in a significant reduction in the level of these compounds in muscle [and other tissues, e.g., the heart (3)], indicating that dietary intake also normally contributes to this reservoir (7).

Given this newly appreciated importance of skeletal muscle in whole-body NO_3^-/NO_2^-/NO metabolism, at least in rodents, it is vital to be able to perform similar measurements in humans, especially in clinical populations that may be deficient in NO, e.g., the elderly (5, 20), patients with heart failure (29), etc. In this context, however, the small size of human muscle biopsy samples represents an analytical challenge, especially if other assays are also to be performed. This is particularly true for NO_2^-, considered the better indicator of NO bioavailability (12, 13), as its concentration in muscle (i.e., 0.5–1 pmol/mg) is much lower than that of NO_3^- (i.e., 100–200 pmol/mg) (7, 22, 23). Laboratory reagents, glassware, etc., are also commonly contaminated with NO_3^- and NO_2^- (9, 15), further compounding the issue.

Numerous methods exist for quantifying NO_3^- and NO_2^- in biological samples (31), several of which should at least theoretically provide sufficient specificity and sensitivity for analysis of small amounts of human muscle. For example, the limit of detection (LOD) of the ozone-based chemiluminescent method used by Piknova et al. (7, 22–24) has been reported to be as low as 0.1 pmol (6). Assuming that the NO_2^- concentration of human muscle is similar to that of rodents, even 1 mg of tissue should be sufficient. Achieving this level of sensitivity may require careful attention to experimental details, however, as the LOD of the chemiluminescent approach is more typically stated to be ~1 pmol (18, 24, 25), requiring a correspondingly greater amount of tissue.

Chemical ionization-gas chromatography mass spectrometry (CI-GCMS) represents another alternative for measuring NO_3^- and NO_2^- in biological samples (30). As reviewed by Tsikas (31), CI-GCMS can provide superb specificity and sensitivity, with an LOD in the femtomole range (30). A disadvantage to this approach, however, is the high cost of the required instrumentation, i.e., 5–10 times that of a chemiluminescent analyzer. As such, a CI-GCMS system may be beyond the reach of an individual laboratory or investigator. Perhaps because of this, to our knowledge, this method has not been used to measure NO_3^- and NO_2^- in either animal or human muscle.

HPLC can also be used to quantify NO_3^- and NO_2^- in blood and tissue (10). NO_3^- and NO_2^- may be detected natively based on chemiluminescence, ultraviolet absorption, or electrochemical methods or after pre- or

postcolumn derivatization to form compounds that are highly fluorescent or absorbent in the visible light range. Depending upon the exact approach, the LOD has been reported to be as low as 0.05 pmol, with methods relying upon derivatization providing the greatest sensitivity (10). HPLC systems are also reasonably affordable, being roughly similar in cost to a chemiluminescent analyzer. However, a potential disadvantage of HPLC in the present context is that reagents commonly used when extracting small metabolites from muscle tissue (e.g., trichloroacetic acid) may interfere with the analysis or even damage the HPLC system.

The purpose of the present study was to develop a method for measuring NO_3^- and NO_2^- in small, i.e., biopsy-sized, muscle samples using a commercial, turnkey HPLC system. Special attention was paid to maximizing recovery of these metabolites from the tissue while avoiding artifactual formation of NO_2^- during sample processing or storage. Although developed for HPLC and with human muscle biopsy samples in mind, the approach may also be useful when samples are analyzed using other methods, e.g., chemiluminescence or CI-GCMS, or whenever the quantity of available tissue is limited, e.g., when studying muscle NO_3^- /NO_2^- /NO metabolism using genetically engineered mice.

METHODS

HPLC System

This study made use of a dedicated HPLC system for measuring NO_3^- and NO_2^- in biological samples with a claimed LOD of 0.1 pmol, i.e., 0.01 µmol/l × 10 µl injected (ENO-30, Eicom USA, San Diego, CA). In this system, NO_3^- and NO_2^- are isolated from each other and from potentially interfering substances on a polystyrene gel separation column, the NO_3^- is quantitatively converted to NO_2^- on a cadmium reduction column, and both are derivatized with Griess reagent followed by spectrophotometric detection at 540 nm. The HPLC was calibrated before each use by manually injecting standards containing known amounts of NO_3^- and NO_2^-.

Source of Tissue and Extraction Methods Tested

All study procedures were approved by the Institutional Animal Care and Use Committee at Indiana University-Purdue University Indianapolis. Male Sprague Dawley rats weighing 300–350 g were anesthetized with isoflurane, euthanized by exsanguination, and the soleus muscle rapidly excised, frozen in liquid N_2, and stored at −80°C. The soleus, which is almost exclusively slow-twitch (1), was chosen to minimize variability between samples because of the varying admixture of muscle fiber types. On the day of analysis, duplicate samples were cut from the midbelly of each muscle while it was still frozen on dry ice and processed using one of four different methods.

Mechanical homogenization plus ultrasound. Per the HPLC manufacturer's suggested guidelines, frozen tissue samples (~50 mg; $n = 4$) were quickly weighed and then homogenized for 10 s in ice-cold HPLC-grade methanol (2:1) using an Omni TH tissue homogenizer (Omni International, Kennesaw, GA) set at maximum speed (35,000 revolutions/min). To further disrupt the tissue (3), each sample was then sonicated for 10 × 1 s using a Fischer Model 120 Sonic Dismembrator fitted with a 1/8th inch probe (Thermo Fischer Scientific, Waltham, MA). Tubes were immersed in an ice bath throughout processing to minimize sample heating. Following sonication, samples were centrifuged at 10,000 *g* for 10 min at 4°C to remove precipitated proteins, the supernatant was transferred to a clean (i.e., NO_2^- - and NO_3^- -free) microcentrifuge tube, and 10 µl were injected into the HPLC.

Bead homogenization. Bead homogenization was performed using a Bullet Blender (Next Advance, Troy, NY). Frozen muscle samples (~50 mg; $n = 6$) were quickly weighed and then placed into ice-cold microcentrifuge tubes preloaded with zirconium oxide beads, and 500 µl of ice-cold methanol were added. Samples were then agitated for 3 min on a speed setting of 12, cooled on ice for 1 min, and this cycle repeated a total of 10 times. After centrifugation at 10,000 *g* for 10 min at 4°C, the supernatant was collected and used for NO_2^- and NO_3^- measurement as described above.

Pulverization at liquid N_2 temperature. Frozen muscle samples (5–10 mg; $n = 6$) were powdered at liquid N_2 temperature in a stainless steel tissue pulverizer (Bessman Tissue Pulverizer, Thermo Fischer Scientific) using several hammer blows to the pestle. The frozen muscle powder was then transferred using a liquid N_2-cooled spatula to a preweighed microcentrifuge tube containing 50 µl of ice-cold methanol. After vortexing, samples were placed on ice for 30 min, quickly reweighed to determine the amount of tissue added, then centrifuged and processed as described above.

Pulverization plus Triton X-100. Frozen muscle samples ($n = 14$) were processed as described immediately above, except that 0.5% Triton X-100 was added to the methanol to help disrupt cell membranes (4).

Recovery Experiment

Recovery of NO_3^- and NO_2^- was determined by powdering ~100 mg of muscle and then extracting 5–10 mg aliquots, as described above, after adding 1,250/12.5, 2,500/25, or 3,750/37.5 pmol of NO_3^-/NO_2^- ($n = 3$ each) to yield final concentrations ~2×, ~3×, and ~4× normal. Percent recovery was calculated after accounting for the contribution of endogenous NO_3^- and NO_2^- based on analysis of unspiked aliquots ($n = 3$) of the same muscle powder.

Effect of Storage

Samples ($n = 6$) were processed using pulverization plus Triton X-100 as described above, and the NO_3^- and NO_2^- content was measured immediately after extraction and again after 15 or 35 days of storage at −80°C. These data were compared with those from samples ($n = 8$) handled identically but with the inclusion of 0.1 mmol/l of oxypurinol in the methanol/Triton X-100 extraction medium to block residual XOR activity (14, 23).

Data Analysis

Analysis of chromatograms was performed using the manufacturer's EPC-700 Envision software. The LOD and limit of quantification (LOQ) were calculated as 3 and 10 times, respectively, the standard error of the estimate of the linear regression of the calibration curve (34). Statistical analyses were performed using GraphPad Prism version 7.02 (GraphPad Software, La Jolla, CA). The effects of the extraction method on NO_3^- content, NO_2^-/NO_3^- ratio, and between-sample reproducibility [i.e., average coefficient of variation (CV) for NO_3^- and NO_2^- content] were determined using one-way ANOVA. The effects of oxypurinol were assessed using two-way (i.e., treatment × time) ANOVA, with time as a repeated measure. Post hoc testing was performed using Fisher's least-significant difference test. The within-day CV was calculated from triplicate injections of the samples used in the recovery experiment. The between-day CV was calculated from the data for the oxypurinol-treated samples in the long-term storage experiment. Data are presented as mean ± SE.

RESULTS

HPLC operating characteristics. The precise response factor (i.e., slope of the calibration curve) varied somewhat (i.e., ~10%) from day to day, depending in part upon the freshness of the Griess reagent and, for NO_3^-, the age of the reduction column. However, repeated calibrations ($n = 5$) demonstrated high linearity (i.e., average $r = 0.9995 \pm 0.0001$ and 0.9996 ± 0.0001 for NO_2^- and NO_3^- standard curves, respectively) and excellent sensitivity (i.e., LOD and LOQ = 0.022 ± 0.001 and 0.073 ± 0.003 pmol for NO_2^- and 0.22 ± 0.01 and 0.74 ± 0.04 pmol for NO_3^-). Representative standard curves for NO_2^- and NO_3^- are shown in Fig. 1.

Neither Triton X-100 nor oxypurinol had any acute influence on retention times, peaks shapes, or peak areas of NO_3^-/NO_2^- standards (data not shown), demonstrating that these reagents did not interfere with the separation or reduction columns or with the Griess reaction. However, the use of Triton X-100 over several days led to mild fronting of the NO_3^- peak, which was reversible by cleaning the separation column per the manufacturer's directions.

Effect of extraction method. Samples prepared via mechanical homogenization followed by ultrasound had the lowest NO_3^- content (Fig. 2, *top*). On the other hand, the ratio of NO_2^- to NO_3^- was significantly elevated (Fig. 2, *bottom*), suggestive of a reduction of NO_3^- to NO_2^- during sample processing. There was also large variability between paired samples from the same muscle (Table 1). Bead beating or pulverization yielded slightly, albeit not significantly, higher NO_3^- levels and a significantly lower NO_2^-/NO_3^- ratio, but reproducibility was still poor. Pulverization followed by extraction with methanol containing 0.5% Triton X-100 resulted in significantly higher values for NO_3^- than the other methods, with the lowest NO_2^-/NO_3^- ratio and the least variability between duplicate samples.

Recovery and reproducibility. Recovery of NO_3^- or NO_2^- added to samples processed using pulverization plus Triton X-100 was high, whereas the CV for repeated measurements of the same sample extract was low, both within and between days (Table 2).

Effect of tissue storage. The NO_3^- content of tissue extracts prepared using pulverization plus Triton X-100 and stored at −80°C was stable for at least 5 wk (data not shown). However, the NO_2^- content, and hence the NO_2^-/NO_3^- ratio, increased essentially linearly over time (Fig. 3). Addition of 0.1 mmol/l oxypurinol to the extraction medium completely blocked this increase, obviating the need to analyze samples immediately after processing.

A representative HPLC chromatogram from a sample prepared using the final method is shown in Fig. 4.

DISCUSSION

Recent studies by Piknova et al. (7, 22, 23) have demonstrated that, at least in rodents, skeletal muscle serves as the body's major NO_3^-/NO_2^- reservoir and thus may be an important source of whole-body NO production via the "reverse" $NO_3^- \rightarrow NO_2^- \rightarrow$ NO pathway. Given the key role of NO in regulating numerous physiological responses, it is critical to be able to perform similar measurements in people, especially in clinical populations [e.g., the elderly (5, 20, 29), patients with heart failure (27), etc.] in whom NO signaling is impaired. To date, however, there is a paucity of reliable data regarding muscle NO_3^- and NO_2^- concentrations in humans. This is especially true for NO_2^-, which is considered the better indicator of NO bioavailability (12, 13). This lack of data stems from the limitations of various methods for quantifying NO_3^- and NO_2^- combined with the small amount of human muscle normally provided by the standard needle biopsy approach.

NO_3^- and NO_2^- can be measured numerous ways (31), with one particularly popular approach in recent years being chemiluminescence (3, 6, 18, 22–25). In this method, NO_3^- or NO_2^- (or nitroso-containing compounds, e.g., *S*-nitrosoglutathione) is reduced offline to form NO, after which the NO is reacted with ozone in a commercial analyzer to generate a detectable photon signal. This approach provides a number of advantages, including relatively low cost and considerable flexibility and selectivity with respect to the chemical species being measured via careful selection of the reducing agent. Chemiluminescence has therefore been used to measure NO_3^- and NO_2^- in numerous tissues (3, 14, 35), including rat skeletal muscle (7, 22, 23). This method was also recently used by Nyakayiru et al. (19) to analyze human muscle biopsy samples. These authors obtained values for NO_3^- but reported that NO_2^- was below the LOD, despite assaying 40 mg of tissue. However, their use of strong acid (i.e., 2% perchloric acid) to deproteinize the samples likely resulted in a significant loss of NO_2^- because of its conversion to highly reactive nitrous acid (HNO_2) that then rapidly disproportionated to form NO and NO_3^- (net reaction $3NO_2^- + 2H^+ \rightarrow 2NO + NO_3^- + H_2O$) (26). Some NO_2^- might also have been lost as a result of HNO_2^--mediated nitros(yl)ation of proteins (33). The latter would seem to be especially problematic when analyzing muscle, which has a much higher protein content than plasma or some other tissues, e.g., the brain (2). Finally, Nyakayiru et al.'s (19) inability to detect NO_2^- may also have been partially due to incomplete extraction of the tissue. This is suggested by the fact that the basal muscle NO_3^- concentration they reported was only ~60 pmol/mg, i.e., only one-third to one-half that of rodent muscle (7, 22, 23; present results) and only moderately higher than that of plasma in the same subjects versus several-fold higher in rats and mice (7, 22, 23).

To avoid such issues, we developed a method to quantify the NO_3^- and NO_2^- concentration of biopsy-sized samples of rat soleus muscle using a commercial HPLC system. The instrument we used relies on the Griess reaction, i.e., the reaction of NO_2^- with sulfanilamide and *N*-(1-naphthyl)ethylenediamine to form a highly absorbent azo dye. This reaction is highly specific for NO_2^- but is notoriously sensitive to interfering substances (e.g., cysteine) common in biological samples (32, 33). This may explain the questionable values for muscle NO_3^- and NO_2^- previously obtained by Montes de Oca et al. in patients with chronic obstructive pulmonary disease (17) and smokers without chronic obstructive pulmonary disease (16) using a batch (microplate) version of the Griess reaction. By first isolating NO_2^- (and NO_3^-) on a separation column, however, such problems can be avoided (32). Indeed, the system we used proved to be highly stable (i.e., normal baseline noise = 0.0002 mV), linear, and sensitive, with a resulting LOD and LOQ for NO_2^- of 0.022 and 0.073 pmol, respectively. At the same time, however, use of this HPLC system introduced additional complications. In particular, the system is incompatible with certain reagents, including strong acids such as those typically used to extract small metabolites from muscle. Thus, a different approach for processing the tissue had to be employed, regardless of any concerns about acid-induced loss of NO_2^- as described above.

As an alternative to strong acid, we relied on methanol to deproteinize the muscle samples, as recommended by the HPLC manufacturer and as previously used by others when analyzing muscle samples for NO_3^- and NO_2^- using the same instrument (21). We were concerned, however, that by itself this "softer" treatment of the tissue might not provide quantitative recovery of these ions, as has been shown for amino acids in the presence of higher protein concentrations (27). We were also concerned about the possibility of artifactual formation of NO_2^- during sample processing because of the reduction of NO_3^- by, e.g., deoxyhemoglobin or deoxymyoglobin (22). When using the chemiluminescent method, this problem can be avoided by first oxidizing heme groups using a potassium ferricyanide-containing "stop" solution (24), but this reagent is also incompatible with the HPLC system. Along with the use of methanol, we tested various mechanical and chemical methods of disrupting the tissue. Our goal was to maximize the yield of NO_3^- and NO_2^- and the

reproducibility of the measurements while minimizing any increase in the NO_2^-/NO_3^- ratio.

Initially, we tried simply mechanically homogenizing samples in methanol, followed by sonication (ultrasound) to further disrupt membranes. This method, however, resulted in low NO_3^- and NO_2^- concentrations, with considerable variability between duplicate samples from the same muscle, presumably because of inadequate disruption of the integrity of the myocytes. The ratio of NO_2^- to NO_3^- was also elevated, suggesting a reduction of the latter to the former during processing, possibly because of local heating during ultrasound treatment. These results are in line with those of Ohtake et al. (21), who found even higher NO_2^-/NO_3^- ratios in the gastrocnemius muscles of KKA^y and C57BL/6J mice using HPLC analysis of methanol-extracted samples.

The next methods we tried were bead homogenization and pulverization at liquid N_2 temperature. These approaches seemed to slightly improve the recovery of NO_3^- and NO_2^- and significantly reduced the NO_2^-/NO_3^- ratio, but reproducibility across duplicate samples was still considered inadequate. Pulverization did, however, permit convenient analysis of much smaller samples than mechanical or bead homogenization. We, therefore, next tested combining pulverization with the use of a detergent (i.e., Triton X-100) to further dissolve/disperse membranes (4). This method significantly increased the yield of NO_3^- and NO_2^- without altering the NO_2^-/NO_3^- ratio and provided better reproducibility across duplicate samples and was therefore chosen for further evaluation.

First, we assessed the recovery of NO_3^- and NO_2^- from samples spiked with known amounts of these compounds, as well as the reproducibility of measurements of the same sample extract. Recovery of NO_3^- and NO_2^- in spiked samples was high, supporting the accuracy of the approach. This conclusion is buttressed by the fact that the muscle NO_3^- and NO_2^- concentrations we obtained using this method are similar to those reported by Piknova et al. (7, 22, 23) for much larger samples analyzed using their chemiluminescent approach. These experiments also demonstrated that our final method provided results that were precise, with both within-day and between-day CVs for both NO_3^- and NO_2^- of <5%. Notably, however, this variability was much lower than that between samples from the same muscle, which averaged $14.0 \pm 4.1\%$ for the duplicate samples used to initially test the method and $11.9 \pm 2.0\%$ for the triplicate samples used in the recovery experiment. Given the high recovery, this between-sample variability appears to have been due to true biological variation and not the result of incomplete extraction of the tissue. As such, these results resemble those obtained for other small muscle metabolites, e.g., lactate, when using acid extraction (11). The physiological significance of this apparently heterogeneous distribution of NO_3^- and NO_2^- in muscle is unknown. It is notable, however, that it was observed even in a muscle, i.e., the soleus, that is composed almost entirely of a single fiber type (1). Given fiber type-related differences in NO synthase expression (28), etc., at least in rats, the variability in NO_3^- and NO_2^- content within and between muscles of varying fiber type seems likely to be considerably larger.

Second, to determine whether deproteinization with methanol was sufficient to eliminate residual XOR activity, we tested the effects of longer-term storage of samples at −80°C. These experiments revealed that although the NO_3^- concentration was stable over time, the NO_2^- concentration, and hence the NO_2^-/NO_3^- ratio, increased in a linear manner. This increase, however, could be completely blocked by inclusion of 0.1 mmol/l oxypurinol in the methanol/Triton X-100 extraction medium, demonstrating that it was not due to the action of any remaining heme groups but more importantly, at least on a practical basis, eliminating the need to extract and analyze samples on the same day.

In summary, we have developed a precise, accurate, and convenient method for extracting and measuring

the NO_3^- and NO_2^- concentration of muscle samples as small as 5 mg using a commercial HPLC system. The theoretical limit is even lower, i.e., 1 mg for both NO_3^- and NO_2^- or 5 ng for NO_3^- alone. This method should prove highly useful in investigating the role of human skeletal muscle in NO_3^-/NO_2^-/NO metabolism in both healthy and diseased subject populations, in response to exercise and dietary interventions, etc. The method should also be useful in other circumstances when the amount of tissue available is limited, e.g., when studying genetically engineered mice. Finally, it should be noted that although we measured NO_3^- and NO_2^- using HPLC, the muscle extraction and storage method that we developed may also be useful when the analysis is performed using a different approach, e.g., chemiluminescence or CI-GCMS. In particular, the use of an organic solvent (i.e., methanol) plus a detergent (i.e., Triton X-100) and an XOR inhibitor (i.e., oxypurinol) instead of a strong acid (e.g., perchloric or trichloroacetic acid) to deproteinize, extract, and preserve muscle samples avoids the potential loss of NO_3^- that may have negatively impacted previous results (19).

GRANTS

This publication was made possible by award numbers R15-HL-121661 and R34-HL-138253 from the National Heart, Lung, and Blood Institute of the NIH.

DISCLAIMERS

The contents of this work are solely the responsibility of the authors and do not necessarily represent the official views of the National Heart, Lung, and Blood Institute or NIH.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

A.D.T., M.B.B., and A.R.C. conceived and designed research; A.D.T., E.J.G., and A.R.C. performed experiments; A.D.T. and A.R.C. analyzed data; A.D.T., M.B.B., and A.R.C. interpreted results of experiments; A.R.C. prepared figures; A.R.C. drafted manuscript; A.D.T., E.J.G., M.B.B., and A.R.C. edited and revised manuscript; A.D.T., E.J.G., M.B.B., and A.R.C. approved final version of manuscript.

REFERENCES

1. Armstrong RB, Phelps RO. Muscle fiber type composition of the rat hindlimb. Am J Anat 171: 259–272, 1984. doi:10.1002/aja.1001710303. [PubMed: 6517030] [CrossRef: 10.1002/aja.1001710303]

2. Banay-Schwartz M, Kenessey A, DeGuzman T, Lajtha A, Palkovits M. Protein content of various regions of rat brain and adult and aging human brain. Age (Omaha) 15: 51–54, 1992. doi:10.1007/BF02435024. [CrossRef: 10.1007/BF02435024]

3. Bryan NS, Fernandez BO, Bauer SM, Garcia-Saura MF, Milsom AB, Rassaf T, Maloney RE, Bharti A, Rodriguez J, Feelisch M. Nitrite is a signaling molecule and regulator of gene expression in mammalian tissues. Nat Chem Biol 1: 290–297, 2005. doi:10.1038/nchembio734. [PubMed: 16408059] [CrossRef: 10.1038/nchembio734]

4. Burden DW. Guide to the disruption of biological samples – 2012. Random Primers 12: 1–25, 2012.

5. Di Massimo C, Lo Presti R, Corbacelli C, Pompei A, Scarpelli P, De Amicis D, Caimi G, Tozzi Ciancarelli MG. Impairment of plasma nitric oxide availability in senescent healthy individuals: apparent involvement of extracellular superoxide dismutase activity. Clin Hemorheol Microcirc 35: 231–237, 2006. [PubMed: 16899934]

6. Feelisch M, Rassaf T, Mnaimneh S, Singh N, Bryan NS, Jourd'Heuil D, Kelm M. Concomitant S-, N-, and heme-nitros(yl)ation in biological tissues and fluids: implications for the fate of NO in vivo. FASEB J 16: 1775–1785, 2002. doi:10.1096/fj.02-0363com. [PubMed: 12409320] [CrossRef: 10.1096/fj.02-0363com]

7. Gilliard CN, Lam JK, Cassel KS, Park JW, Schechter AN, Piknova B. Effect of dietary nitrate levels on nitrate fluxes in rat skeletal muscle and liver. Nitric Oxide 75: 1–7, 2018. doi:10.1016/j.niox.2018.01.010. [PMCID: PMC5860979] [PubMed: 29378248] [CrossRef: 10.1016/j.niox.2018.01.010]

9. Ishibashi T, Himeno M, Imaizumi N, Maejima K, Nakano S, Uchida K, Yoshida J, Nishio M. NO(x) contamination in laboratory ware and effect of countermeasures. Nitric Oxide 4: 516–525, 2000. doi:10.1006/niox.2000.0302. [PubMed: 11020340] [CrossRef: 10.1006/niox.2000.0302]

10. Jobgen WS, Jobgen SC, Li H, Meininger CJ, Wu G. Analysis of nitrite and nitrate in biological samples using high-performance liquid chromatography. J Chromatogr B Analyt Technol Biomed Life Sci 851: 71–82, 2007. doi:10.1016/j.jchromb.2006.07.018. [PubMed: 16904955] [CrossRef: 10.1016/j.jchromb.2006.07.018]

11. Karlsson J, Diamant B, Saltin B. Muscle metabolites during submaximal and maximal exercise in man. Scand J Clin Lab Invest 26: 385–394, 1970. doi:10.3109/00365517009046250. [PubMed: 5486403] [CrossRef: 10.3109/00365517009046250]

12. Kleinbongard P, Dejam A, Lauer T, Rassaf T, Schindler A, Picker O, Scheeren T, Gödecke A, Schrader J, Schulz R, Heusch G, Schaub GA, Bryan NS, Feelisch M, Kelm M. Plasma nitrite reflects constitutive nitric oxide synthase activity in mammals. Free Radic Biol Med 35: 790–796, 2003. doi:10.1016/S0891-5849(03)00406-4. [PubMed: 14583343] [CrossRef: 10.1016/S0891-5849(03)00406-4]

13. Lauer T, Preik M, Rassaf T, Strauer BE, Deussen A, Feelisch M, Kelm M. Plasma nitrite rather than nitrate reflects regional endothelial nitric oxide synthase activity but lacks intrinsic vasodilator action. Proc Natl Acad Sci USA 98: 12814–12819, 2001. doi:10.1073/pnas.221381098. [PMCID: PMC60136] [PubMed: 11606734] [CrossRef: 10.1073/pnas.221381098]

14. Li H, Cui H, Kundu TK, Alzawahra W, Zweier JL. Nitric oxide production from nitrite occurs primarily in tissues not in the blood: critical role of xanthine oxidase and aldehyde oxidase. J Biol Chem 283: 17855–17863, 2008. doi:10.1074/jbc.M801785200. [PMCID: PMC2440597] [PubMed: 18424432] [CrossRef: 10.1074/jbc.M801785200]

15. Makela S, Yazdanpanah M, Adatia I, Ellis G. Disposable surgical gloves and Pasteur (Transfer) pipettes as potential sources of contamination in nitrite and nitrate assays. Clin Chem 43: 2418–2420, 1997. [PubMed: 9439464]

16. Montes de Oca M, Loeb E, Torres SH, De Sanctis J, Hernández N, Tálamo C. Peripheral muscle alterations in non-COPD smokers. Chest 133: 13–18, 2008. doi:10.1378/chest.07-1592. [PubMed: 18187741] [CrossRef: 10.1378/chest.07-1592]

17. Montes de Oca M, Torres SH, De Sanctis J, Mata A, Hernández N, Tálamo C. Skeletal muscle inflammation and nitric oxide in patients with COPD. Eur Respir J 26: 390–397, 2005. doi:10.1183/09031936.05.00107404. [PubMed: 16135718] [CrossRef: 10.1183/09031936.05.00107404]

18. Nagababu E, Rifkind JM. Measurement of plasma nitrite by chemiluminescence without interference of S-, N-nitroso and nitrated species. Free Radic Biol Med 42: 1146–1154, 2007. doi:10.1016/j.freeradbiomed.2006.12.029. [PMCID: PMC2722945] [PubMed: 17382196] [CrossRef: 10.1016/j.freeradbiomed.2006.12.029]

19. Nyakayiru J, Kouw IWK, Cermak NM, Senden JM, van Loon LJC, Verdijk LB. Sodium nitrate ingestion increases skeletal muscle nitrate content in humans. J Appl Physiol (1985) 123: 637–644, 2017. doi:10.1152/japplphysiol.01036.2016. [PubMed: 28663382] [CrossRef: 10.1152/japplphysiol.01036.2016]

20. Nyberg M, Blackwell JR, Damsgaard R, Jones AM, Hellsten Y, Mortensen SP. Lifelong physical activity prevents an age-related reduction in arterial and skeletal muscle nitric oxide bioavailability in humans. J Physiol 590: 5361–5370, 2012. doi:10.1113/jphysiol.2012.239053. [PMCID: PMC3515824] [PubMed: 22890714] [CrossRef: 10.1113/jphysiol.2012.239053]

21. Ohtake K, Nakano G, Ehara N, Sonoda K, Ito J, Uchida H, Kobayashi J. Dietary nitrite supplementation improves insulin resistance in type 2 diabetic KKA(^{y}) mice. Nitric Oxide 44: 31–38, 2015. doi:10.1016/j.niox.2014.11.009. [PubMed: 25461271] [CrossRef: 10.1016/j.niox.2014.11.009]

22. Piknova B, Park JW, Lam KK, Schechter AN. Nitrate as a source of nitrite and nitric oxide during exercise hyperemia in rat skeletal muscle. Nitric Oxide 55-56: 54–61, 2016. doi:10.1016/j.niox.2016.03.005. [PMCID: PMC4860042] [PubMed: 27000467] [CrossRef: 10.1016/j.niox.2016.03.005]

23. Piknova B, Park JW, Swanson KM, Dey S, Noguchi CT, Schechter AN. Skeletal muscle as an endogenous nitrate reservoir. Nitric Oxide 47: 10–16, 2015. doi:10.1016/j.niox.2015.02.145. [PMCID: PMC4439352] [PubMed: 25727730] [CrossRef: 10.1016/j.niox.2015.02.145]

24. Piknova B, Schechter AN. Measurement of nitrite in blood samples using the ferricyanide-based hemoglobin oxidation assay. Methods Mol Biol 704: 39–56, 2011. doi:10.1007/978-1-61737-964-2_4. [PMCID: PMC3489475] [PubMed: 21161628] [CrossRef: 10.1007/978-1-61737-964-2_4]

25. Pinder AG, Rogers SC, Khalatbari A, Ingram TE, James PE. The measurement of nitric oxide and its metabolites in biological samples by ozone-based chemiluminescence. Methods Mol Biol 476: 11–28, 2008. [PubMed: 19157006]

26. Samouilov A, Kuppusamy P, Zweier JL. Evaluation of the magnitude and rate of nitric oxide production from nitrite in biological systems. Arch Biochem Biophys 357: 1–7, 1998. doi:10.1006/abbi.1998.0785. [PubMed: 9721176] [CrossRef: 10.1006/abbi.1998.0785]

27. Sedgwick GW, Fenton TW, Thompson JR. Effect of protein precipitating agents on the recovery of plasma free amino acids. Can J Anim Sci 71: 953–957, 1991. doi:10.4141/cjas91-116. [CrossRef: 10.4141/cjas91-116]

28. Stamler JS, Meissner G. Physiology of nitric oxide in skeletal muscle. Physiol Rev 81: 209–237, 2001. doi:10.1152/physrev.2001.81.1.209. [PubMed: 11152758] [CrossRef: 10.1152/physrev.2001.81.1.209]

29. Tang L, Wang H, Ziolo MT. Targeting NOS as a therapeutic approach for heart failure. Pharmacol Ther 142: 306–315, 2014. doi:10.1016/j.pharmthera.2013.12.013. [PubMed: 24380841] [CrossRef:

10.1016/j.pharmthera.2013.12.013]

30. Tsikas D. Simultaneous derivatization and quantification of the nitric oxide metabolites nitrite and nitrate in biological fluids by gas chromatography/mass spectrometry. Anal Chem 72: 4064–4072, 2000. doi:10.1021/ac9913255. [PubMed: 10994966] [CrossRef: 10.1021/ac9913255]

31. Tsikas D. Methods of quantitative analysis of the nitric oxide metabolites nitrite and nitrate in human biological fluids. Free Radic Res 39: 797–815, 2005. doi:10.1080/10715760500053651. [PubMed: 16036360] [CrossRef: 10.1080/10715760500053651]

32. Tsikas D. Analysis of nitrite and nitrate in biological fluids by assays based on the Griess reaction: appraisal of the Griess reaction in the L-arginine/nitric oxide area of research. J Chromatogr B Analyt Technol Biomed Life Sci 851: 51–70, 2007. doi:10.1016/j.jchromb.2006.07.054. [PubMed: 16950667] [CrossRef: 10.1016/j.jchromb.2006.07.054]

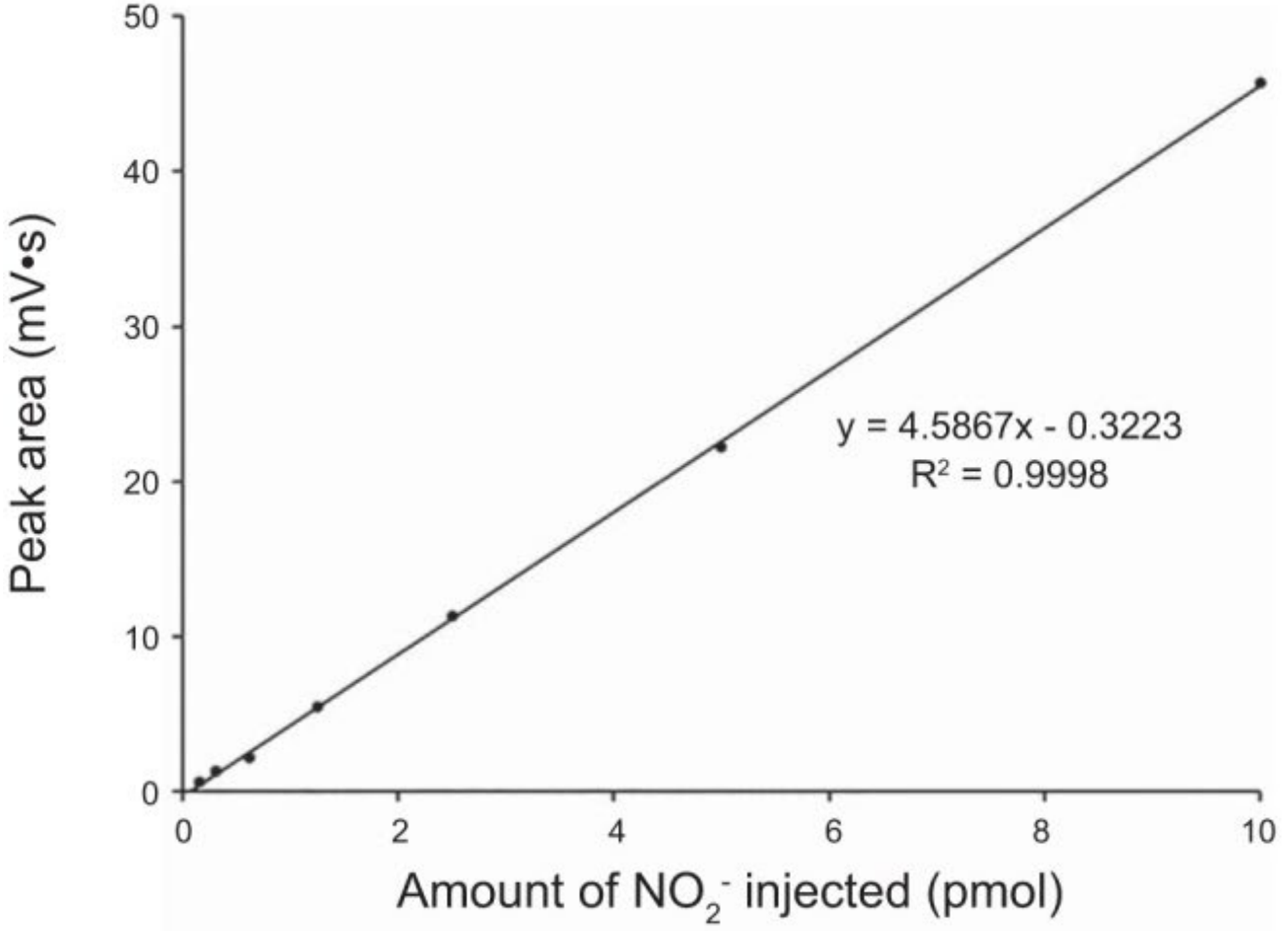
33. Tsikas D, Gutzki FM, Rossa S, Bauer H, Neumann C, Dockendorff K, Sandmann J, Frölich JC. Measurement of nitrite and nitrate in biological fluids by gas chromatography-mass spectrometry and by the Griess assay: problems with the Griess assay–solutions by gas chromatography-mass spectrometry. Anal Biochem 244: 208–220, 1997. doi:10.1006/abio.1996.9880. [PubMed: 9025936] [CrossRef: 10.1006/abio.1996.9880]

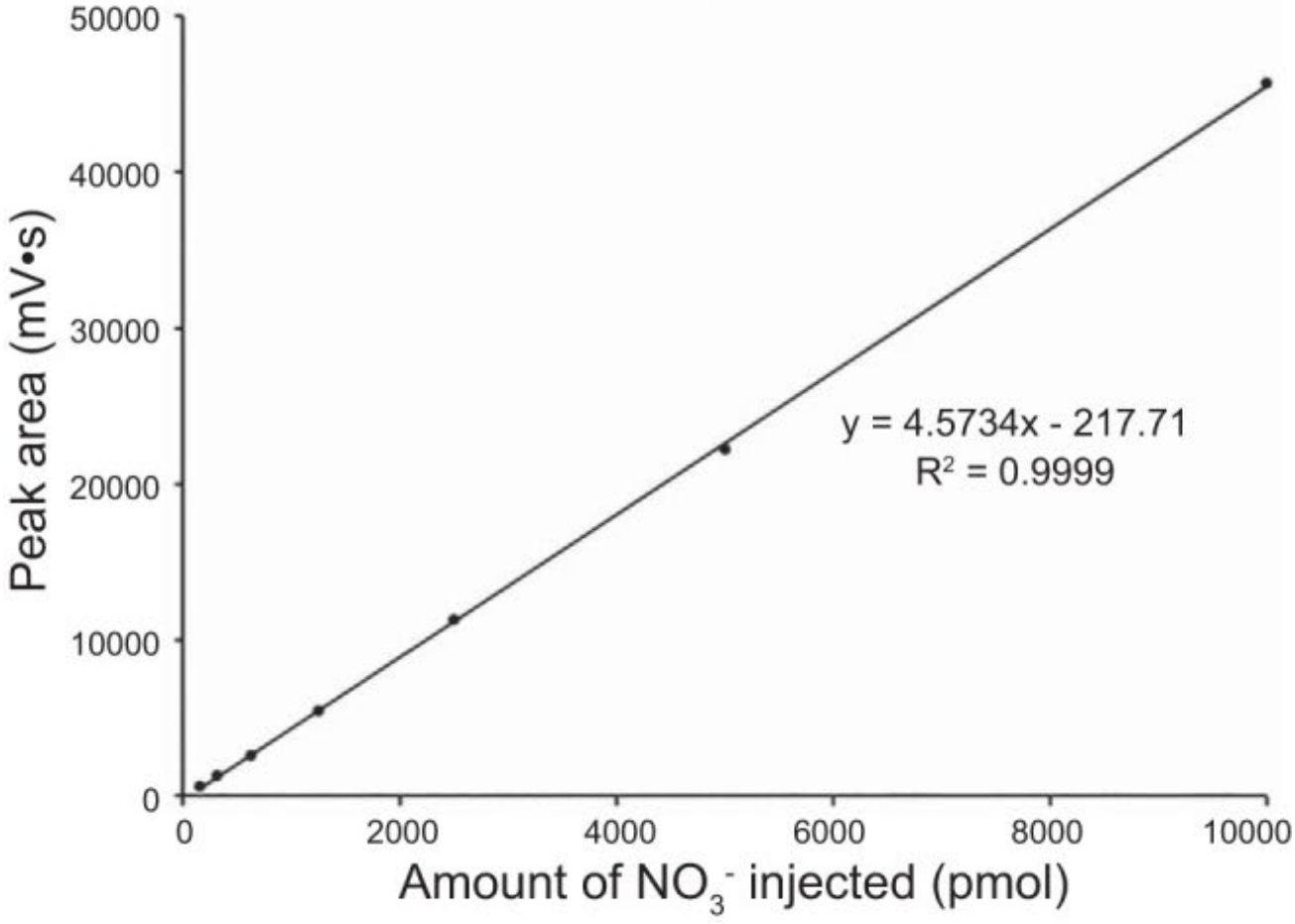
34. Validation of Analytical Procedures: Text and Methodology. Guidance for Industry: Q2 (R1). Geneva: International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH), 2005.

35. Zweier JL, Li H, Samouilov A, Liu X. Mechanisms of nitrite reduction to nitric oxide in the heart and vessel wall. Nitric Oxide 22: 83–90, 2010. doi:10.1016/j.niox.2009.12.004. [PMCID: PMC2851168] [PubMed: 20044016] [CrossRef: 10.1016/j.niox.2009.12.004]

Figures and Tables

Fig. 1.

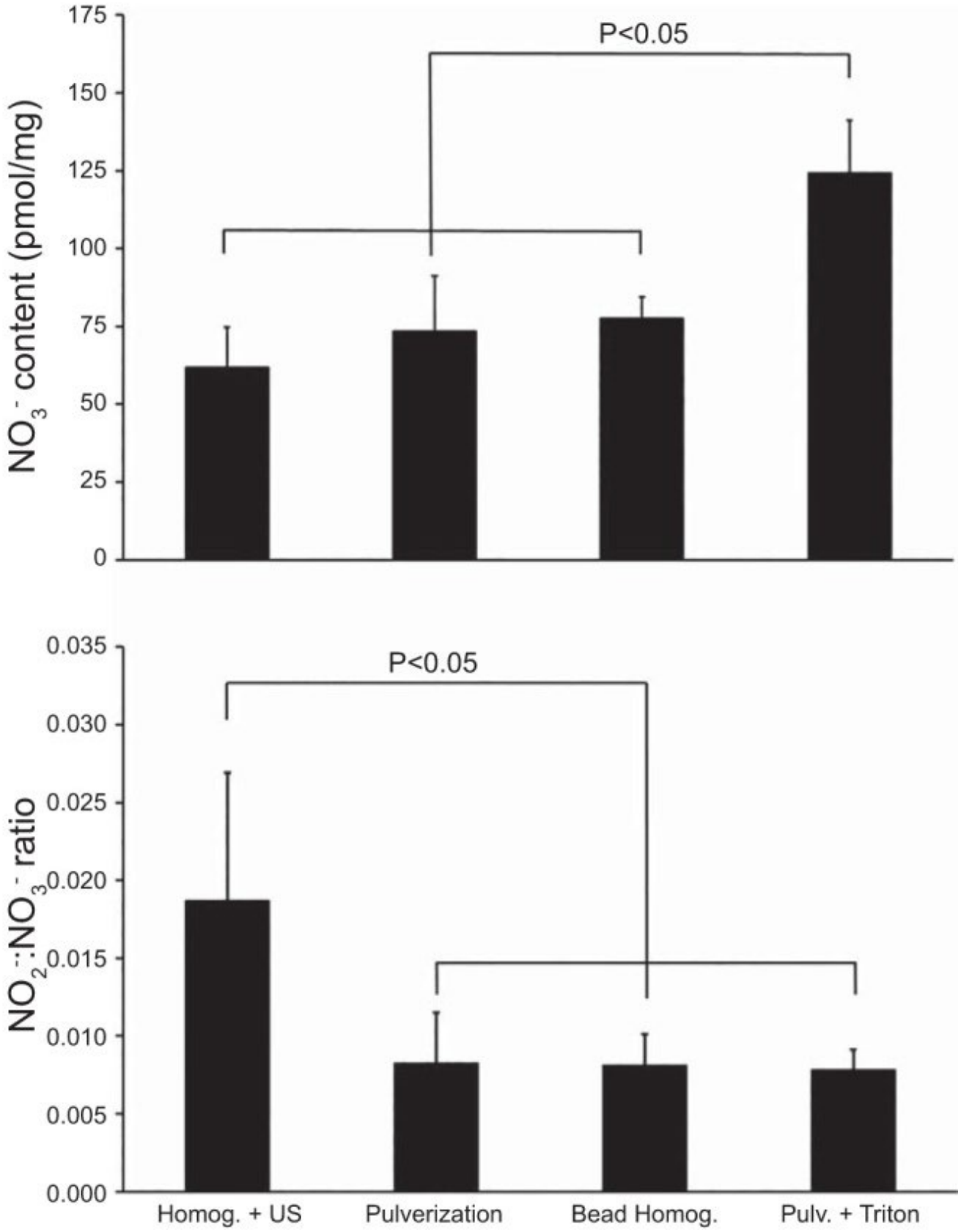




Open in a separate window

Representative nitrite (NO_2^-) and nitrate (NO_3^-) standard curves.

Fig. 2.



Nitrate (NO_3^-) content (*top*) and nitrite (NO_2^-)/NO_3^- ratio (*bottom*) of rat soleus muscles processed by mechanical homogenization plus ultrasound ($n = 4$), pulverization at liquid N_2 temperature ($n = 6$), bead homogenization ($n = 6$), or pulverization plus 0.5% Triton X-100 ($n = 14$). Data are means ± SE. Significance of differences determined by one-way ANOVA. Homog, homogenization; Pulv, pulverization; US, ultrasound.

Table 1.

Coefficients of variation for analysis of replicate samples from the same muscle

	Mechanical Homogenization + Ultrasound	Pulverization	Bead Homogenization	Pulverization + 0.5% Triton X-100
NO_2^-, %	45.7 ± 33.3	18.4 ± 12.0	9.7 ± 6.7	16.7 ± 6.0
NO_3^-, %	58.0 ± 23.6	51.6 ± 23.9	39.0 ± 16.0	11.3 ± 3.7*
Average, %	51.8 ± 28.4	35.0 ± 9.1	24.3 ± 10.3	14.0 ± 4.1†

Values are mean ± SE for $n = 4$–14 samples. NO_2^-, nitrite; NO_3^-, nitrate.

*Significantly different from mechanical homogenization + ultrasound or pulverization ($P < 0.05$), †significantly different from mechanical homogenization + ultrasound ($P < 0.05$).

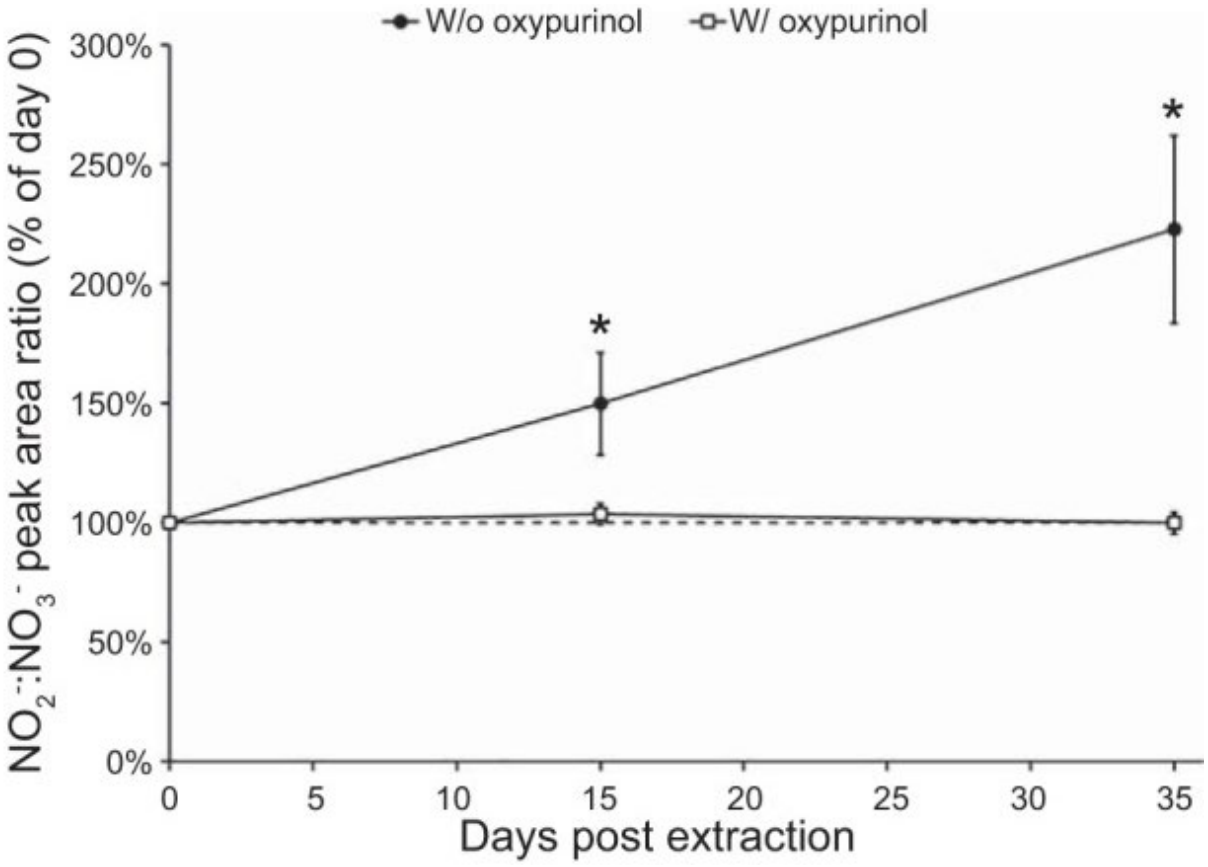
Table 2.

Recovery and reproducibility for samples processed using pulverization plus 0.5% Triton X-100

	NO_2^-	NO_3^-
Recovery, %	86.0 ± 3.1	96.0 ± 4.9
Within-day CV, %	3.7 ± 0.7	3.0 ± 0.4
Between-day CV, %	4.4 ± 1.0	3.5 ± 0.5

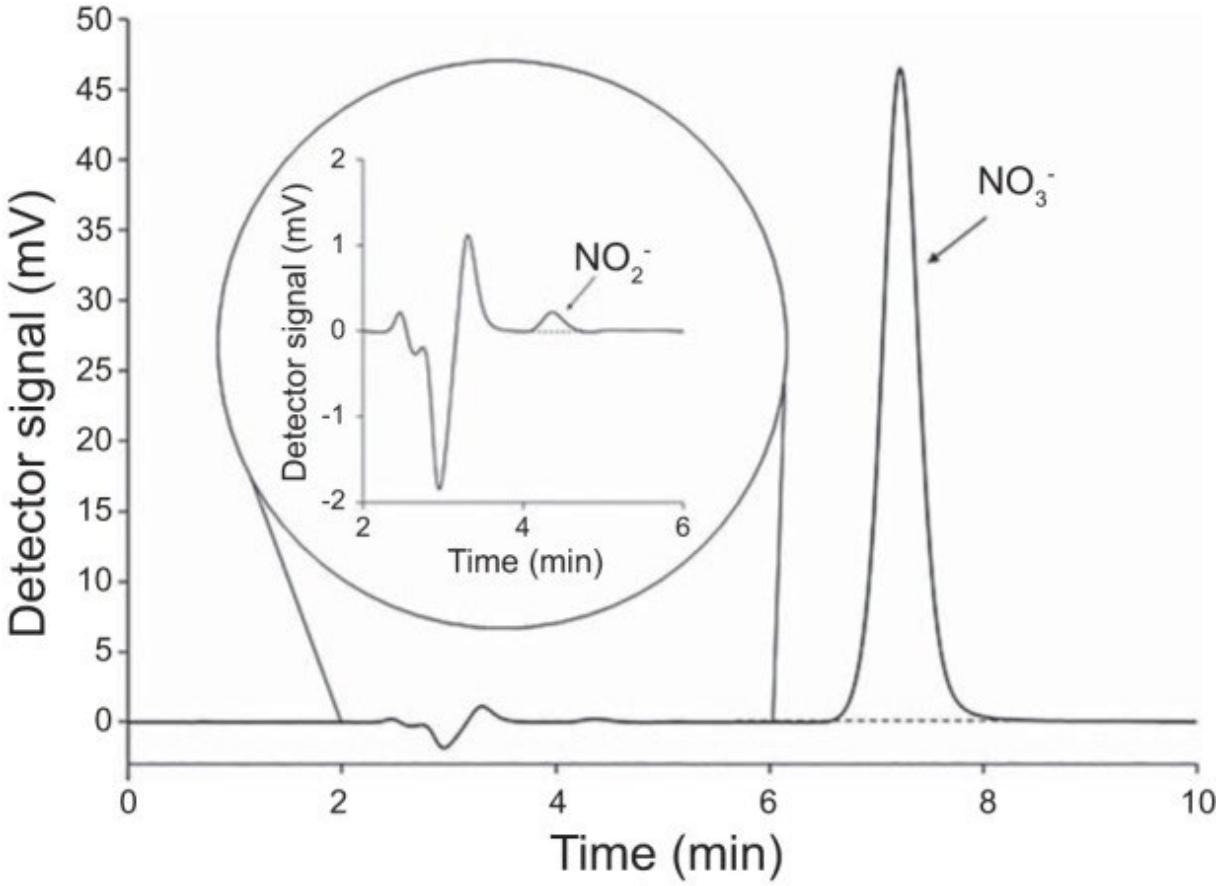
Values are mean ± SE for $n = 8$–12 samples. CV, coefficient of variation; NO_2^-, nitrite; NO_3^-, nitrate.

Fig. 3.



Effect of addition of 0.1 mmol/l oxypurinol to the methanol plus 0.5% Triton X-100 extraction medium on the nitrite (NO_2^-)/nitrate (NO_3^-) ratio of samples stored at −80°C for up to 5 wk. *$P < 0.01$ versus *day 0* value for same samples. Data are means ± SE for $n = 6$ samples without (w/o) oxypurinol and $n = 8$ for samples with (w/) oxypurinol. Significance of differences determined by two-way (i.e., treatment × time) ANOVA, with time as a repeated measure.

Fig. 4.



Sample HPLC chromatogram showing nitrite (NO_2^-) (*inset*) and nitrate (NO_3^-) peaks from 6.75 mg of rat soleus muscle processed by pulverization at liquid N_2 temperature followed by extraction with 50 µl of methanol containing 0.5% Triton X-100 and 0.1 mmol/l oxypurinol. Ten-microliter injection. Measured NO_2^- content = 0.482 pmol/mg; NO_3^- content = 111.1 pmol/mg.